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Detection of *Candida vulvovaginitis* in Clinical Samples; Using Direct Polymerase Chain Reaction Without DNA Extraction

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Background: Vulvovaginal candidiasis (VVC) is a common disease which infects women. The current study investigated the performance of direct sample polymerase chain reaction (DS-PCR) method to detect *Candida* spp. in clinical samples of vulvovaginitisto compare the results to those of standard microbiological laboratory methods.
Objectives: The current study aimed to further simplify the DNA extraction procedure, and shorten the time required for isolation and identification by using direct PCR to identify *Candida vulvovaginitis* without DNA extraction from samples or colonies.
Patients and Methods: In the current study, totally 150 sexually active women participated. Vaginal discharge samples were collected using two sterile Dacron swabs that were immediately placed in two tubes each containing 1 mL of distilled water. One of the tubes was used for conventional culture methods whereas the other one was used for DS-PCR without DNA extraction. The number of yeast cells in each sample was counted.
Results: The results showed that out of the 150 samples, 55 were positive and 63 samples were negative by both methods, and 32 samples were positive using the culture method, but negative by DS-PCR. All positive DS-PCR samples had $> 10^7$ yeast or conidia cells/mL. The sensitivity and specificity of DS-PCR were calculated as 63.2% and 100%, respectively.
Conclusions: Direct sample PCR has the potential to rapidly and accurately diagnose *Candida vulvovaginitis* in patients, especially if sufficient samples are obtained.

Keywords: Candida; Direct Sample PCR

1. Background

Vulvovaginal candidiasis (VVC) is a common disease which infects women and may affect their physical and emotional health, as well as relationships with their sexual partners. Conventional methods to identify *Candida* species are based on culture, assimilation, fermentation reactions, and morphology. However, these techniques are time-consuming and their reliance on phenotypic expression makes them potentially unreliable. To overcome the many limitations of phenotypic methods involve diagnosis of vulvovaginal candidiasis, recent advances in molecular DNA analysis have facilitated the development of identification systems at the species level (1). DNA isolation and purification is a key step for most protocols in molecular biological studies, including polymerase chain reaction (PCR). Various methods proposed that extraction and purification of yeast DNA can be classified according to the following cells lysis techniques: bead beating, enzymatic cell wall lyses using lyticase, or cell permeabilization using chaotropic agents. However, these techniques are generally very time-consuming. Moreover, some protocols require additional lyses steps, such as mechanical disruption and sonification, or the use of toxic substances such as phenol-chloroform solution (2). In essence, these approaches are no faster than conventional approaches because first, the organism must be isolated in pure culture (3).

In contrast, a number of reports have described the PCR amplification of purified DNA extracted from clinical *Candida* specimens (2); however, the drawback to these approaches is the additional time for genomic DNA extraction required for the amplification of specific genes.

2. Objectives

The current study aimed to further simplify the DNA extraction procedure and shorten the time required for isolation in culture and identification by using direct PCR to identify *Candida vulvovaginitis* without DNA extraction.

3. Patients and Methods

3.1. Clinical Samples

One hundred and fifty cases examined for inclusion

criteria of vulvovaginal candidiasis (age 15 to 50, non-pregnant, without drug use, with severe itching and abnormal vaginal discharge). In their routine clinical laboratory diagnosis, all vaginal specimens were collected in the health and medical clinics and hospitals of Qazvin University of Medical Sciences (Qazvin, Iran) from March to December 2012. Vaginal discharge samples were collected with sterile Dacron swabs and then deposited into two tubes, each containing 1mL of sterile distilled water. Yeast cells were counted in each sample (using a hemocytometer); one of the tubes was used for standard conventional phenotypic and mycological methods and the other for direct sample PCR (DS-PCR), in which aliquots of the samples were directly used as templates that opposed to the conventional templates of purified extracted genomic DNA for routine PCR.

3.1.1.Phenotypic and Mycological Evaluations

Phenotypic and mycological evaluations were performed by direct slide preparations as well as germ tube, chlamydospore formation, and specially culture in chrome agar to detect color of the colonies (4-6).

3.1.2. Sensitivity Detection Limit and Determination of Minimum Yeast Concentration for Performing DS-PCR

Serial dilutions (10^{10}–1) of *C. albicans* cells (ATCC 10231) were prepared (enumerated microscopically using a hemocytometer). Five microliters of each suspension was used as DS-PCR template.

3.2. Primers

The following universal (pan fungal) primers were synthesized by metabion international AG. (Planegg-Martinsried, Germany):

a) ITS1 or F (5'-TCC GTA GGT GAA CCT GCG G-3');
b) ITS4 or R (5'-TCCTCC GCT TAT TGA TAT GC-3') (1, 2, 7-9).

3.3. Polymerase Chain Reaction

For the present comparative study, DS-PCR was performed for each of the 150 clinical samples. Optimal PCR conditions were as follows: a reaction volume of 25 μL contained 10 μL of master mix (Amplicon, Denmark), 1 μL of each F and R primers, 5 μL of the sample as a direct template, and remaining volume (8 μL) of distilled water (DW). A negative control containing sterile deionized water and a positive control of *C. albicans* DNA (ATCC 10231) were included in each PCR run. Reaction mixtures were subjected to an initial denaturation step at 94°C for seven minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for one minute, and extension at 72°C for one minute, with a final extension at 72°C for seven minutes (1, 2, 8) in Veriti Thermal Cycler ABI (applied biosystems, Foster City, CA, USA).

3.4. Electrophoresis Analysis

The PCR products were subjected to 2% agaros gel electrophoresis and the gels were stained with gel red (power load). A 100-basepair DNA ladder (Intron Biotechnology Inc.) was used for all experiments.

3.5. Statistical Methods

The data were analyzed using SPSS software, version 16 (SPSS Inc., Chicago, IL). Cross tabulations, and 2×2 contingency tables were used to calculate sensitivity and specificity with a 95% confidence interval. The test sensitivity of the DS-PCR was defined as the number of strains correctly identified in the conventional culture as the gold standard method (true positives) divided by the total number of isolated yeast strains. The test specificity was defined as the number of strains which were not identified by DS-PCR (true negatives) divided by the total number of strains.

4. Results

The mean age of the participants was 32 ± 10.5 years (range, 21.5–42.5 years).

4.1. Detection of the sensitivity of Direct Sample Polymerase Chain Reaction or analytical sensitivity

Electrophoresis of the direct PCR products of serially diluted yeast cells of 10^7 cells/mLor more, revealed a visible band (in between of 510 to 870 bp (Figure 1), suggesting that the simulated patient samples containing the abovementioned number of yeasts are detectable; therefore, actual samples with these yeast concentrations should be detectable through this method.

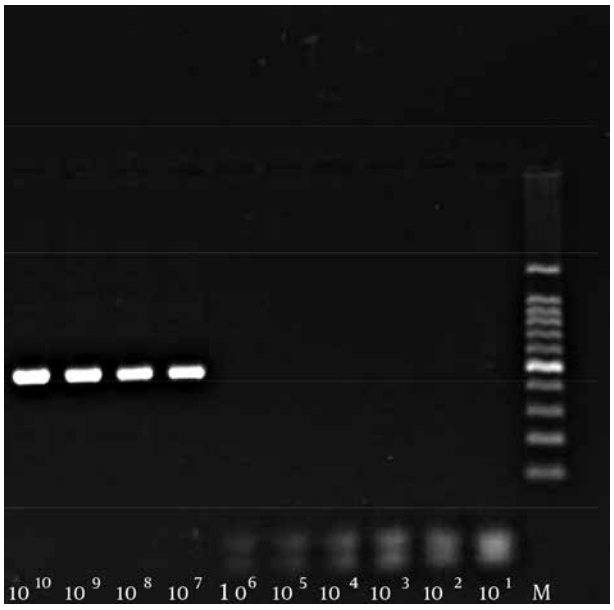


Figure 1. Agarose Gel Electrophoresis of DS-PCR of Serially Diluted Yeast Cells

4.2. Comparison between suggested Polymerase Chain Reaction and Mycological Methods to Detect Candida vulvovaginitis

Of the 150 samples, 55 were positive by both methods (DS-PCR and mycological methods), 32 were positive by mycological evaluations, but negative by DS-PCR and 63 cases were negative by both methods. In total, from 87 *Candida* species identified through mycological methods, 73 *C. albicans*, 12 *C. glabrata* and two *C. tropicalis* were identified.

The constructed tables of TP (true positive), TN (true negative), FP (false positive), and FN (false negative) results, from which test sensitivity, test specificity, PPV and NPV with 95% confidence intervals were calculated as follow:

4.2.1. Sensitivity

Ability of DS-PCR to diagnose the vulvovaginitis was 63.2%, which is relatively acceptable.

4.2.2. Specificity

The Ability of DS-PCR to exclude vulvovaginitis was excellent. It means that the accuracy of DS-PCR to correctly diagnose vulvovaginitis was 100%.

4.2.3. PPV (Positive Predictive Value)

Extent to which a positive DS-PCR indicates vulvovaginitisin a given population was excellent (100%).

4.2.4. NPV (Negative Predictive Value)

Extent to which a negative DSPCR exclude the vulvovaginitis in a given population was fair (66.3%) (Table 1).

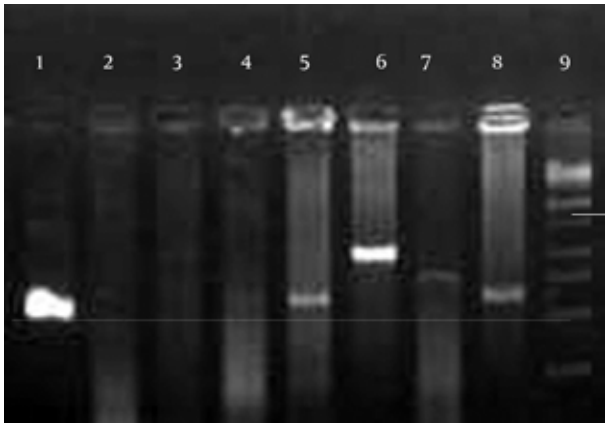
All DS-PCR-positive samples had more than 10^7 yeast cells per mL (55 cases), but 32 positive culture and negative DS-PCR samples contained less than 107 yeast cells per mL, and did not reveal visible electrophoretic bands (Figure 2).

Table 1. Performance Characteristics of DS-PCR Test for Detection of *Candida* to Detect Vulvovaginitis (n = 150) [a,b,c]

DS DS-PCR (New Test)	Culture Mycological Methods (Gold Method)	
	Negative (n = 63)	**Positive (n = 87)**
Positive (n = 87)	FP = 0	TP = 55
Negative (n = 63)	TN = 63	FN = 32

[a] Abbreviations: DS-PCR, direct sample polymerase chain reaction; FP, false-positive; FN, false-negative; TN, true-negative; TP, true-positive.
[b] Test sensitivity: TP/ (TP+FN) × 100 = 55/ (55+32) × 100 = 63.2%; Test specificity: TN/ (TN+FP) × 100 = 63/ (63+0) × 100 = 100%; PPV: 55/ (55+0) × 100 = 100%.
[c] NPV: 63/ (63+32) × 100 = 66.3%.

Figure 2. Agarose Gel Electrophoresis to compare two methods for detection of Candida Vulvovaginitis



(Lanes 1, positive control of C. albicans; 2, negative control of DW; 3 and 4, negative DS-PCR and mycological diagnosed samples, respectively; 5 and 7, positive mycological sample and the same sample for DS-PCR, respectively; 6, another positive control of C. glabrata; 8, a positive DS-PCR sample, and 9a 100 bp size marker).

5. Discussion

It is generally accepted that DNA extraction from yeasts, either by enzymatic lysis or by bead sonication followed by phenol-chloroform extraction, is the most tedious and cumbersome step of a PCR-based identification method (10) and additional time to culture the clinical specimens and colony isolation, limits its routine use in the clinical laboratories (2). This is the first study which describes a rapid and reliable detection and identification method for clinically relevant *Candida* species directly from vulvovaginitis samples, which can aid clinicians to the faster and more reliable method, without need for growing the microorganisms or performing additional laboratory procedures, such as DNA extraction.

Previous studies regarding the evaluation of DC-PCR (i.e. DC-PCR) to detect *Candida* species concluded that it is feasible to start PCR directly from *Candida* colonies without DNA of colonies to be extracted (2, 11). Furthermore, other studies to the detection of *Candida* species and fungemia directly from blood cultures vials by PCR methods, reported that it is possible to detect DNA of organisms in the patients` blood samples without the need for culture and colony formation (10, 12, 13).

By combining these findings, the current study examined the applicability of DS-PCR to detect *Candida* species in vulvovaginitis, using direct samples on clinical swabs (colonization of *Candida* species in such situations results in true in-vivo colonies rather than presence of single cell *Candida* in normal flora samples).

Undoubtedly, fungal cell breakage and subsequent release of genomic DNA in these conditions are less efficient than the preliminary true extraction of DNA. However, in ideal vulvovaginal sampling, adequate DNA templates are available to yield positive PCR tests (2).

Also, intact yeast cells are steadily amplified, probably

because in perfect vulvovaginal sampling, numerous cells are collected and the amplified rDNA are present in multiple copies (> 100) per genome (14). Therefore, it seems possible that during the various thermal PCR cycles the release of DNA from yeast cells will yield sufficient templates (intact DNA) to promote PCR amplification of the abundant rDNA in the yeast genome. This is why some DNA extraction protocols and kits employ only the boiling and thermal steps for an efficient lysis step (10). The use of FTA-card system (Whatman Inc., Piscataway, NJ, USA) to instantly lyse yeast cells after heating can produce sufficient yeast DNA templates for PCR. Chang et al. (10) reported that the only step used for DNA extraction with their recommended kit (microlysis) was heating of yeast cell suspensions in the lysis solution in a thermal cycler ; thereby, eliminating the use of phenol-chloroform and alcohol for DNA purification and precipitation, respectively.

It is obvious that among the most important factors that limit the application of DS-PCR to amplify *Candida* DNA in vaginal samples is the concentration of yeast cells in the samples which must be more than 10^7/mL. Therefore, to achieve precise results, a high yeast content sample and experienced technologists are required; otherwise, false-negative (FN) results and low sensitivity of the test may occur. Therefore, to reduce FN results and improve test sensitivity as well as NPV, counting of yeast cells in the samples and having concentrations above 10^7/mL is mandatory. In fact, Mirhendi et al. (2) reported that at least 10^6 *Candida* cells are needed to perform their recommended DC-PCR (direct colony PCR). There were no false positive (FP) results in our suggested DS-PCR method which meant that every positive result of this technique indicated vulvovaginitis in patients.

Another important limiting factor of DS-PCR, as well as conventional PCR, to detect *Candida* species in vaginal samples is the use of universal primers to amplify the highly conserved 5.8 S region of rDNA and its adjacent ITS1 and ITS4sequences in *Candida* species (15), which may have similarities with other eukaryotic rDNAs, including those from humans and other fungi. For example, other yeasts such as Geothrichum and especially Trichosporon species are sometimes isolated from vaginal infection (16, 17) and PCR amplification by ITS1 and ITS4 primers for these fungi may yield amplicon sizes in the ranges of *Candida* species (9). Although size determination of the PCR products gives a possible species, subsequent restriction analysis and PCR melting profile (PCR MP) as well as ITS region nucleotide sequencing lead to accurate diagnosis of the relevant yeasts at the species levels (1, 14, 18, 19).

Although there were no such yeasts in the cultures of the samples of the current study, it is recommended that in the future, to perform DS-PCR, such similarities should be evaluated. If these sequences match human or other fungi DNA-derived sequences, new *Candida*-specific primers (20) must be designed to permit sole detection of the major clinically important species present in the specimens.

In conclusion the DS-PCR method has the potential to rapidly and accurately diagnose *Candida vulvovaginitis* and the samples obtained directly from patients may be preferred to mycological laboratory methods and/or DNA extracted samples, since they can provide an opportunity for a rapid and direct PCR. In future, the current study authors plan to study the application of this method for other pathogenic fungi, including medically important moulds present in other clinical specimens. However, to release DNA from such fungi samples and obtain good results, a prior digestion step using lyticase enzyme is recommended.

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Authors' Contributions

Dr. Sarookhani: designing of the project and corresponding author; Sohrabi: mycological evaluations; Ezani: performing PCR.

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